British Journal of Pharmacology (1997) 120, 1483–1490

The isoquinoline derivative KN-62 a potent antagonist of the P2Z-receptor of human lymphocytes

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1 Extracellular adenosine 5′-triphosphate (ATP) is an agonist for a P2Z receptor on human lymphocytes which mediates opening of a cation-selective ion channel, activation of phospholipase D and shedding of the adhesion molecule, L-selectin, from the cell surface. The isoquinolinesulphonamides, KN-62, (1-[*N*, *O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine), a selective antagonist of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and KN-04, (N-[1-[N-methyl-p-(5 isoquinoline sulphonyl)benzyl]-2-(4 phenylpiperazine)ethyl]-5-isoquinolinesulphonamide) an inactive analogue, were used to investigate the possible role of CaMKII in these diverse effects of extracellular ATP.

2 KN-62 potently antagonized ATP-stimulated Ba^{2+} influx into fura-2 loaded human lymphocytes with an IC_{50} of 12.7 ± 1.5 nM ($n=3$) and complete inhibition of the flux at a concentration of 500 nM. Similarly, KN-62 inhibited ATP-stimulated ethidium$^+$ uptake, measured by time resolved flow cytometry, with an IC_{50} of 13.1 ± 2.6 nM ($n=4$) and complete inhibition of the flux at 500 nM.

3 KN-04 antagonized ATP-stimulated Ba^{2+} influx with an IC_{50} of 17.3 ± 2.7 nM ($n=3$). Similarly, KN-04 inhibited ATP-stimulated ethidium$^+$ uptake with an IC_{50} of 37.2 ± 8.9 nM ($n=4$). Both fluxes were completely inhibited at 500 nM KN-04.

4 ATP-stimulated phospholipase D activity, measured in [^{3}H]-oleic acid-labelled lymphocytes by the transphosphatidylation reaction, was antagonized by KN-62 and KN-04, with 50% inhibition at 5.9 ± 1.2 and 9.7 ± 2.8 nM ($n=3$), respectively. Both KN-62 and KN-04 inhibited ATP-stimulated shedding of L-selectin, measured by flow cytometric analysis of cell surface L-selectin, with IC_{50} values of 31.5 ± 4.5 and 78.7 ± 10.8 nM ($n=3$), respectively. Neither of the isoquinolinesulphonamides (500 nM) inhibited phorbol ester- or ionomycin-stimulated phospholipase D activity or phorbol ester-induced shedding of L-selectin.

5 The inhibitory effect of KN-62 or KN-04 on P2Z-mediated responses was slow in onset (5 min) and only partially reversed by washing the cells.

6 Both KN-62 and KN-04 (at 500 nM) had no effect on uridine 5′-triphosphate (UTP)-stimulated Ca^{2+} transients in fura-2 loaded human neutrophils, a response which is mediated by the $P2Y_2$ receptor.

7 Thus, KN-62 and KN-04 are potent antagonists of the P2Z receptor and at nanomolar concentrations inhibit all known responses mediated by the P2Z receptor of human lymphocytes. In contrast, KN-62 and KN-04 had no effect on responses mediated by the $P2Y_2$ receptor of neutrophils. Moreover, since KN-62 and KN-04 are almost equipotent, the P2Z-mediated responses do not involve CaMKII, but indicate that the isoquinolinesulphonamides are potent and direct inhibitors of the P2Z-receptor.

Keywords: P2Z receptor; KN-62; KN-04; lymphocytes (human leukaemic); isoquinolinesulphonamide; extracellular ATP receptor; antagonist; cation channel; lymphocyte; phospholipase D; $P2Y_2$ receptor; L-selectin

Introduction

Extracellular adenosine 5′-phosphate (ATP) mediates a wide range of effects by acting on P2-receptors expressed on many tissues throughout the body. P2-receptors have been classified into 2 classes, P2X and P2Y, based on molecular structure of the cloned receptors and mechanism of signal transduction (Fredholm *et al*., 1994; Burnstock, 1996). Within the P2Y and P2X receptor families are a number of subtypes, designated $P2Y_1-P2Y_7$ and $P2X_1-P2X_6$, based on structure and pharmacological profiles of agonist and antagonist responses (Burnstock, 1996). Thus the P2U receptor is now classed as a $P2Y_2$ receptor. Many cell types express the G-protein coupled, P2Y receptors, whose occupancy activates the phosphoinositide specific phospholipase C signalling cascade and raises cytosolic [Ca^{2+}] by releasing Ca^{2+} from internal stores (Dubyak & El-Moatassim, 1993). The P2X receptors are ligand gated cation channels which conduct Ca^{2+} from the extracellular medium. The P2Z receptor, which may constitute a third class of P2-receptor (Fredholm *et al*., 1994), is expressed on cells of immune and haemopoietic origin. The P2Z receptor is also a ligand-gated cation channel with a distinct agonist profile, but its function is less clear (Wiley & Dubyak, 1989; Wiley *et al*., 1990; 1992; Dubyak & El-Moatassim, 1993). Some evidence suggests that the P2Z receptor may mediate apoptosis of rat thymocytes and murine lymphocytes and macrophages, since extracellular ATP causes influx of Ca^{2+}, release of lactic dehydrogenase (LDH) and cytolysis (Zheng *et al*., 1991; Di Virgilio, 1995). In human leukaemic lymphocytes, we have shown multiple effects of extracellular ATP mediated via occupancy of the P2Z receptor by the agonist, fully ionized ATP^{4-} species. Thus extracellular ATP increases the permeability of lymphocytes to Ca^{2+}, Ba^{2+} and ethidium$^+$ (Wiley *et al*., 1990; 1993; 1994), stimulates phospholipase D activity (Gargett *et al*., 1996) and induces shedding of L-selectin (Jamieson *et al*., 1996). Recently, a P2Z receptor has been cloned from rat brain and the first 395 amino acids shown to have 35–40% homology with P2X receptors. This cloned receptor, designated $P2X_7$ on the basis of this homology, contains a long carboxyl terminal domain not found in other P2X subtypes which confers the unique permeability of P2Z-channels to large cations such as fluorescent dyes (Surprenant *et al*., 1996).

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At present, there are no specific antagonists of the P2Z receptor which are active in the nanomolar range. The best known inhibitor of P2-receptors is suramin, which inhibits not only the lymphocyte P2Z receptor (IC_{50} 60 μM) but also P2X and P2Y receptors (Leff *et al*., 1990; Wiley *et al*., 1993). Suramin also inhibits receptors for several growth factors (eg bFGF, PDGF, IL-3 etc) and a wide variety of enzymes, including ecto-ATPase (Voogd *et al*., 1993; Crack *et al*., 1995). Oxidised ATP (2,3 dialdehyde ATP) inhibits the P2Z receptor but its effect is irreversible and requires exposure of cells to high concentrations of inhibitor (ca 300 μM for 60 min) (Murgia *et al*., 1993; Wiley *et al*., 1994). Amiloride analogues, such as hexamethylene amiloride (HMA), are the most potent known inhibitors of the P2Z-receptor with IC_{50}s of 2–10 μM, but at maximal effective concentrations produce only incomplete block (70–85%) of cation fluxes through the P2Z ion channel (Wiley *et al*., 1996). The problems of antagonist potency and non-selectivity are further compounded by the coexpression of several P2 receptor subtypes on many cells. Thus mast cells express P2Y and P2Z receptors (Cockcroft & Gomperts; 1979; Osipchuck & Cahalan, 1992) and macrophages P2Y and P2Z (Nuttle *et al*., 1993). However, lymphocytes from patients with chronic lymphocytic leukaemia only express P2Z receptors and since this receptor has been well characterized (Wiley *et al*., 1990; 1992; 1993; 1994), lymphocytes are an ideal cell type in which to evaluate the effect of antagonists.

In order to determine whether P2Z-stimulated responses in human lymphocytes were dependent on calmodulin-dependent protein kinase II (CaM-kinase II), a study was made of the isoquinolinesulphonamide, KN-62, a selective and potent inhibitor (IC_{50} 0.9 μM) of the kinase (Tokumitsu *et al*., 1990; Hidaka & Kobayashi, 1992). A structural analogue, KN-04, which has no effect on CaM-kinase II at 100 μM (Ishikawa *et al*., 1990) was used as a control compound. In this paper, we present the unexpected finding that both KN-62 and KN-04 are potent inhibitors of P2Z-receptor mediated effects and are active in the low nanomolar range.

Methods

Source of lymphocytes

Peripheral blood lymphocytes were obtained from eight separate patients with B-cell chronic lymphocytic leukaemia whose cells showed permeability responses to ATP in our previous studies (Wiley *et al*., 1993; 1994; Gargett *et al*., 1996).

Lymphocyte preparation

Venous blood (20 ml) from patients was added to heparin anticoagulant and diluted with 2 vol of HEPES buffered saline (composition mM: HEPES 10, pH 7.4, NaCl 145, KCl 5, $CaCl_2$ 1, D-glucose 5 and bovine serum albumin (BSA) 1 g l^{-1}). Mononuclear cells were separated by density gradient centrifugation over Ficoll-Paque and washed twice. Cytocentrifuge preparations showed that >99% of cells were small mature lymphocytes.

Neutrophil preparation

Venous blood (50 ml) from normal human donors was added to heparin anti-coagulant and mononuclear cells removed as described above. The red cell pellet was resuspended in 5% dextran T-500 in saline to the original volume and the red cells allowed to sediment for 30 min at 20°C. The leukocyte rich supernatant was removed, washed twice in HEPES buffered saline, contaminating red blood cells were removed by hypotonic lysis in 135 mM ammonium chloride buffered with 17 mM Tris, pH 7.4 and the remaining neutrophils washed twice in HEPES-buffered saline. Cytocentrifuge preparations showed that >99% of cells were neutrophils.

Cytosolic Ca^{2+} and Ba^{2+} measurements by fluorimetry

Washed lymphocytes or neutrophils (1×10^7 ml^{-1}) were suspended in HEPES buffered saline and were loaded with 2 μM fura-2-acetoxymethyl ester by incubation at 37°C for 20 min in the dark. Cells were then washed twice with HEPES buffered saline. Cells (1×10^8 ml^{-1}) were kept in the dark at 20°C and lymphocytes were diluted to 2.0×10^6 ml^{-1} in 3 ml of 150 mM KCl medium containing HEPES 10 mM, pH 7.4, BSA 1 g l^{-1} and D-glucose, while neutrophils were diluted to 1.0×10^6 ml^{-1} in 3 ml of Ca^{2+}-free HEPES buffered saline. Both cell suspensions were incubated with or without KN-62 or KN-04 (1 nM–1 μM) for 5 min at 37°C before fluorimetric analysis in a stirred cuvette at 37°C. $BaCl_2$ (0.5 mM), $CaCl_2$ (1 mM) or EGTA (0.2 mM) and ATP were added as indicated in the continued presence of inhibitor or diluent. Changes in cytosolic Ca^{2+} or Ba^{2+} were monitored by a Johnson Foundation Fluorometer with excitation at 340 nm and emission at 500 nm. Calibration of F_{max} and F_{min} was performed after each run by adding digitonin (25 μg ml^{-1}, final concentration) followed by EGTA (6 mM final concentration in 50 mM Tris, pH 8.5) (Grynkiewicz *et al*., 1985).

Ethidium influx measurement by flow cytometry

Lymphocytes (1×10^8 ml^{-1}) were diluted to 1.0×10^6 ml^{-1} in 1 ml of 150 mM KCl medium containing HEPES 10 mM, pH 7.4, BSA 1 g l^{-1} and D-glucose 5 mM. Cell suspensions were incubated with or without KN-62 or KN-04 (1 nM–1 μM) for 5 min at 37°C, followed by ATP (500 μM) and incubated a further 2 min before the addition of ethidium (25 μM). Fluorescent signals were collected from stirred and temperature controlled (37°C) samples 30 s before and up to 5 min after ethidium addition in the continued presence of inhibitor or diluent. Histograms (256 channels) of lymphocyte associated fluorescence signals were collected over consecutive 6 s intervals with a Coulter Elite flow cytometer (Coulter, Hialeah, FL) with an argon laser excitation at 488 nm. Fluorescent emission was collected with a 590 nm long-pass filter. The mean channel of fluorescence intensity was then calculated for each of the histograms collected for consecutive 6 s intervals and plotted against time.

Phospholipase D assay

Lymphocytes (1×10^7 ml^{-1}) were cultured with [^{3}H]-oleic acid (2–5 μCi ml^{-1}, specific activity 10 Ci $mmol^{-1}$) for 20–24 h in RPMI-1640 medium supplemented with gentamicin (40 μg ml^{-1}), 10% heat inactivated foetal calf serum (FCS) at 37°C to label membrane phospholipids. Labelled cells were washed twice in HEPES buffered saline followed by a final wash in either HEPES buffered saline or 150 mM KCl medium containing HEPES 10 mM, pH 7.4, bovine serum albumin (BSA) 1 g l^{-1} and D-glucose 5 mM and $CaCl_2$ 1 mM. Three ml aliquots containing 1.1×10^7 ml^{-1} lymphocytes were warmed to 37°C and incubated with or without KN-62 or KN-04 (1 nM–500 nM) for 5 min, then 900 μl aliquots were added to 100 μl butanol (final concentration 30 mM) for a further 5 min, and stimulated with 1 mM ATP for 15 min with gentle mixing in the continued presence of inhibitor or diluent. The phospholipase D reaction was terminated by addition of 1 ml of 20 mM $MgCl_2$ followed by centrifugation and addition of 1 ml ice cold methanol. Membrane lipids were extracted into chloroform/HCl at 4°C under N_2 as described previously (Gargett *et al*., 1996), and separated by silica gel thin layer chromatography (t.l.c.) with the solvent system, ethyl acetate/iso-octane/acetic acid/water (13:2:3:10, v/v) under saturating conditions. Sample spots were located by autoradiography and [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) spots identified by an authentic standard. [^{3}H]-PBut and [^{3}H]-phospholipid spots were scraped into scintillant fluid (PPO in toluene, 4 g l^{-1}) and counted in a liquid scintillation counter. The quantity of [^{3}H]-PBut is presented as a percentage of total ^{3}H labelled-cellular

phospholipids. Phospholipase D assays were performed in triplicate and data are expressed as the mean ± s.e.mean.

Immunofluorescent staining for L-selectin

Lymphocytes (1.0×10^6 ml^{-1}) were suspended in 150 mM KCl medium containing HEPES 10 mM pH 7.4, BSA 1 g l^{-1} and D-glucose and 3 ml aliquots were incubated with or without KN-62 or KN-04 (1 nM – 500 nM) for 5 min, followed by a 5 min incubation with ATP (500 μM) in the continued presence of inhibitor or diluent. The incubations were ended by addition of 3 ml 20 mM $MgCl_2$ and cells were pelleted and resuspended in 100 μl of medium and 5 μl Fluorescein labelled CD62L monoclonal antibody (anti L-selectin) was added. After 10 min at 20°C, cells were washed once and resuspended in 1 ml medium and analysed on a Coulter Elite flow cytometer (Coulter, Hialeah, FL) with an argon laser excitation at 488 nm and a 530 nm band-pass emission filter. Non-specific staining was analysed by incubating cells for 15 min at 37°C with an equivalent amount of non-immune immunoglobulin (Coulter Electronics) in place of the L-selectin antibody. The mean channel of fluorescence of 5,000 cells was collected by linear amplification.

Calculations of ATP^{4-} species concentration

The ATP concentration required to achieve a constant ATP^{4-} concentration (260 μM) in all experiments was calculated by an updated version (3.0) of a previous published programme, Bound and Determined (Brooks & Storey, 1992).

Materials

Ficoll-Paque (density 1.077) was obtained from Pharmacia (Uppsala, Sweden). ATP, BzATP, ethidium bromide, barium chloride, phorbol ester (phorbol 12-myristate 13-acetate (PMA)) and bovine serum albumin (BSA) were from Sigma Chemical Co. (St Louis, MO). Roswell Park Memorial Institute-1640, glutamine and foetal calf serum (FCS) were obtained from Flow laboratories, North Ryde, Australia. [9,10-^{3}H]-oleic acid was from Amersham International (U.K.) and t.l.c. plates (LK6D) were from Whatman, (Maidstone, U.K.). Fura-2-acetoxymethyl ester was from Molecular Probes (Eugene, OR). HEPES and Tris were from Boehringer Mannheim (Germany). L-selectin monoclonal antibody (CD62L) was from the DREG.55 clone from Bender Medsystems (Vienna, Austria). Dextran T-500 was from Pharmacia Biotech (Uppsala, Sweden). KN-62 (1-[*N*, *O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine) was from Research Biochemicals, Inc. (Natick, MA) and KN-04 (N-[1-[N-methyl-p-(5-isoquinolinesulphonyl)benzyl]-2-(4 phenylpiperazine)ethyl]-5-isoquinoline sulphonamide) was from Seikagaku (Tokyo, Japan). Stock solutions of KN-62 and KN-04 were made in DMSO and stored at 4°C. DMSO was present at 0.1 or 0.2% in assays and had no effect on the parameter measured.

Data presentation and analysis

Concentration-inhibition curves were obtained by non linear regression analysis with the programme Flexifit (Guardabasso *et al.*, 1988). Hill slope values were calculated from these curves by use of the Hill equation. Statistical analysis of data was by ANOVA. Data are presented as mean ± s.e.mean (*n*).

Results

KN-62 inhibits ATP-stimulated permeant fluxes in human lymphocytes

Extracellular ATP activates the P2Z receptor of human leukaemic lymphocytes and opens a Ca^{2+}-selective cation channel which also conducts Ba^{2+}. ATP-stimulated Ba^{2+} uptake into cells loaded with fura-2 was measured fluorimetrically, since Ba^{2+} produces changes in the excitation and emission spectra of fura-2 that are almost identical to those produced by Ca^{2+}. Figure 1a shows that addition of 0.5 mM Ba^{2+} to cells suspended in Ca^{2+}-free KCl medium resulted in little change in fura-2 saturation. However, when 640 μM ATP (260 μM ATP^{4-}) was added there was a rapid increase in fura-2 saturation from 0.42 to 0.95 (Figure 1a) as Ba^{2+} entered the cells through the P2Z-operated ion channel. Preincubation of cells with the isoquinolinesulphonamide, KN-62 for 5 min at 37°C produced a concentration-dependent inhibition of ATP-stimulated Ba^{2+} influx which was maximal at 500 nM (Figure 1). Analysis of the initial rates of Ba^{2+} influx (Figure 1b) gave an

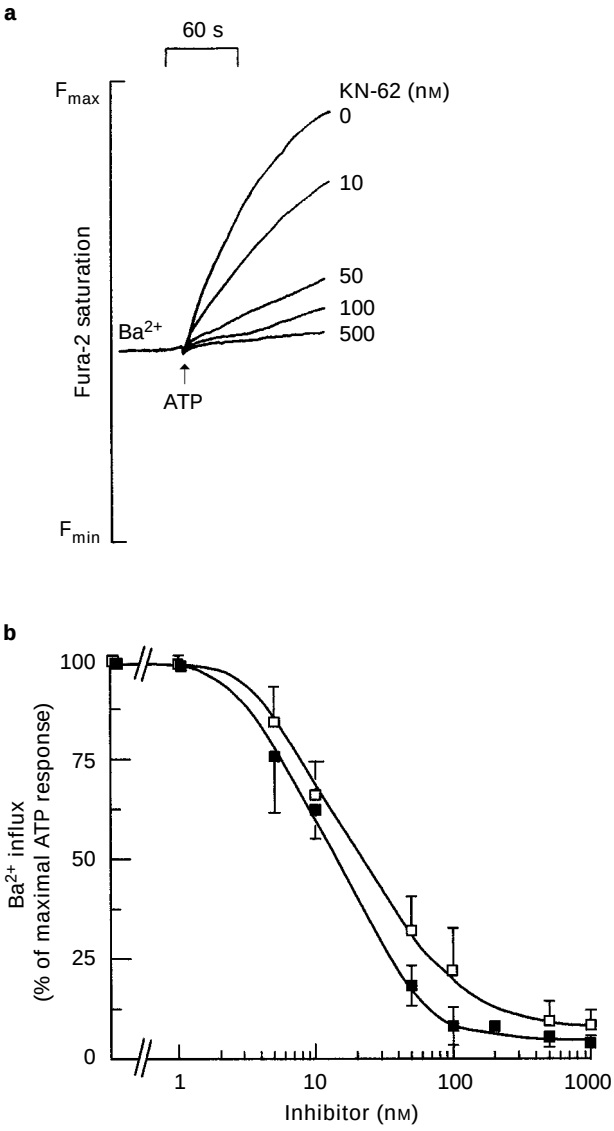

Figure 1 Inhibition of ATP-stimulated Ba^{2+} influx by KN-62 and KN-04. (a) Fura-2 loaded lymphocytes were suspended at 2×10^6 ml^{-1} in HEPES-buffered KCl medium, preincubated with or without KN-62 for 5 min at 37°C in a stirred fluorimeter cuvette, and 0.5 mM Ba^{2+} was added 1 min before stimulation with 640 μM ATP (ATP^{4-} 260 μM) as indicated. (b) Initial rates of ATP-stimulated Ba^{2+} influx were obtained from cells stimulated with ATP in the presence of KN-62 (■) or KN-04 (□) as described above. Results are expressed as a percentage of maximal response to ATP in the absence of inhibitor, which was defined as 100% response. The curves shown were calculated by non linear regression analyses. The data could be well described by Hill analysis which yielded Hill values of -1.16 ± 0.14 (3) and -0.94 ± 0.10 (3) for KN-62 and KN-04, respectively. Mean values from experiments on 3 patients are shown; vertical lines indicate s.e.mean.

IC_{50} of 12.7±1.5 nM for KN-62 and maximal inhibition of 95.9±2.5% (mean±s.e.mean, $n=3$ experiments from 3 patients). Similarly, in fura-2 loaded cells, the ATP-stimulated rise in cytosolic [Ca^{2+}] from a basal of 140 nM to 700 nM was inhibited by KN-62 in a concentration-dependent manner (results not shown). No basal uptake of Ba^{2+} or Ca^{2+} was observed in the presence or absence of 1 μM KN-62.

The fluorescent dye ethidium$^+$ is a permeant for the P2Z-receptor-operated ion channel of lymphocytes and influx of this cation can be measured by time resolved flow cytometry (Wiley *et al*., 1993). Addition of 500 μM ATP (260 μM ATP^{4-}) induced ethidium$^+$ uptake into lymphocytes suspended in a Ca^{2+}-free KCl medium, shown in Figure 2a as an increase in mean channel fluorescence which was linear with time over 5 min. Figure 2a also shows that preincubation of cells with KN-62 for 5 min before the addition of ATP inhibited the ATP-stimulated ethidium influx at all concentrations between 1 and 500 nM. Analysis of KN-62 inhibition of the rates of ethidium$^+$ uptake (Figure 2b) gave an IC_{50} of 13.1±2.6 nM with maximal inhibition of the uptake of 97.7±4.0% at 500 nM KN-62 ($n=4$). There was no basal uptake of ethidium$^+$ into lymphocytes nor did KN-62 (500 nM) increase the low basal permeability to ethidium$^+$.

KN-04 also inhibits ATP-stimulated permeant fluxes

The observed inhibitory effect of KN-62 on ATP-stimulated cation fluxes raised the possibility that CaMKII may be involved in regulating the P2Z-receptor. The inhibitory activity of KN-04 was examined, since this structural analogue of KN-62 has no inhibitory effect on CaMKII at 100 μM (Ishikawa *et al*., 1990). Figure 1b shows that KN-04 potently inhibited ATP-stimulated Ba^{2+} influx in a concentration-dependent manner (IC_{50} of 17.3±2.7 nM) with maximal inhibition of 91.4±2.7% of the flux at a concentration of 500 nM ($n=3$). Similarly, KN-04 potently antagonized ATP-induced ethidium$^+$ uptake (IC_{50} 37.2±8.9 nM) with maximal inhibition of 95.3±5.7% at a concentration of 500 nM ($n=4$) (Figure 2b). These results exclude an involvement of CaMKII in the regulation of the P2Z receptor. The effect of these two isoquinolinesulphonamides on other P2Z-mediated responses in lymphocytes was then studied.

Inhibition by KN-62 and KN-04 of ATP-stimulated phospholipase D activation

To explore the inhibitory specificity of KN-62 and KN-04, the effect of these inhibitors on ATP-stimulated, ionomycin-stimulated and phorbol ester-stimulated phospholipase D activity was examined, since the three agonists stimulate phospholipase D activity through different signal transduction pathways (Exton, 1994). Phospholipase D activity was measured in the presence of butanol, as phospholipase D catalyses the transphosphatidylation reaction producing the stable end product [^{3}H]-PBut. In human leukaemic lymphocytes, ionomycin (5 μM) stimulated an 8 fold increase in [^{3}H]-PBut accumulation over basal, while phorbol ester (100 nM) stimulated a 3.6 fold increase (Figure 3a). Similarly, 1 mM ATP (260 μM ATP^{4-}) increased phospholipase D activity 3.5 fold over basal. However, while both KN-62 (500 nM) and KN-04 (500 nM) completely inhibited ATP-stimulated phospholipase D activity ($P<0.0001$), the inhibitors had no effect on phorbol ester- or ionomycin-stimulated phospholipase D activity (Figure 3a), even at concentrations up to 5 μM of inhibitors (results not shown). The concentration-dependence of phospholipase D inhibition by either KN-62 or KN-04 added 10 min before ATP is shown in Figure 3b. Both compounds antagonized ATP-stimulated phospholipase D activity, over the concentration range 1–500 nM, producing 50% inhibition at 5.9±1.2 and 9.7±2.8 nM ($n=3$), respectively. Neither compound affected basal phospholipase D activity.

KN-62 and KN-04 inhibit other P2Z-mediated responses in lymphocytes

Stimulation of the P2Z receptor by extracellular ATP also causes shedding of the surface adhesion molecule, L-selectin (CD62L) by activation of a membrane proteolytic activity, which is independent of Ca^{2+} influx (Jamieson *et al*., 1996). Cells were preincubated with KN-62 or KN-04 (5–500 nM) for 5 min at 37°C, 500 μM ATP (260 μM ATP^{4-}) was added and after another 5 min the cells were stained by fluorescein-labelled anti L-selectin antibody and analysed by flow cytometry. Again, both KN-62 and KN-04 prevented ATP-

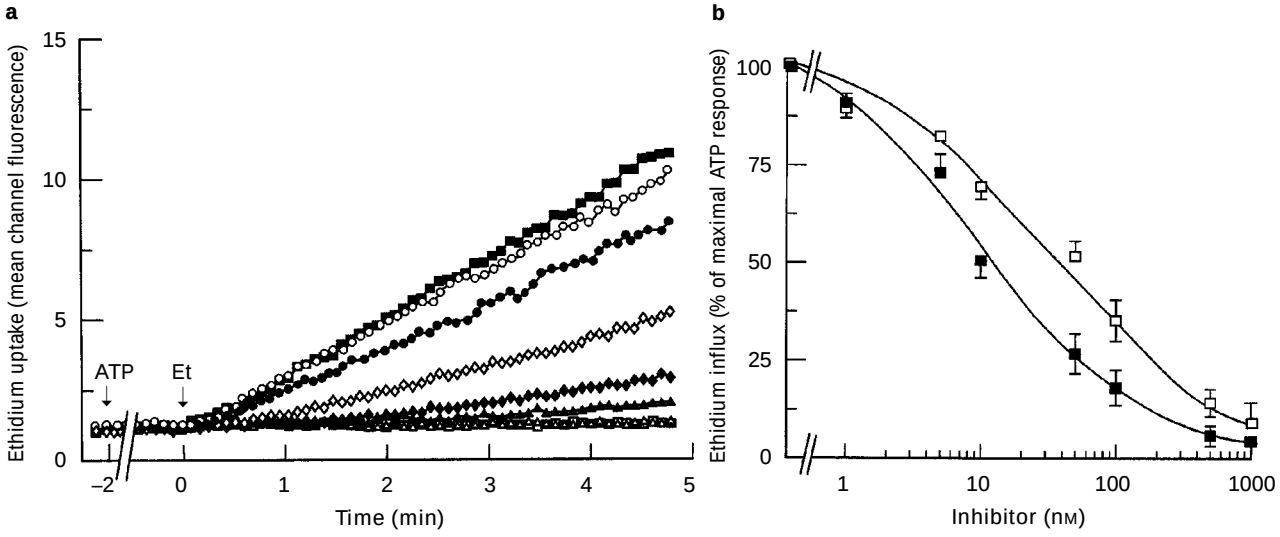

Figure 2 Inhibition of ATP-stimulated ethidium$^+$ influx by KN-62 and KN-04. (a) Lymphocytes, suspended at 10^6 ml^{-1} in Ca^{2+}-free HEPES buffered KCl medium, were preincubated in the absence (■, □) or presence of KN-62: 1 (○), 5 (●), 10 (◇), 50 (◆), 100 (▲) and 500 (△) nM for 5 min at 37°C followed by additions of 500 μM ATP (260 μM ATP^{4-}) and 25 μM ethidium$^+$ as indicated. Mean channel cell associated fluorescence was measured at 6 s intervals by flow cytometry. (b) Initial rates of ATP-stimulated ethidium$^+$ influx were obtained from cells stimulated with ATP in the presence of KN-62 (■) or KN-04 (□) as described above. Results are expressed as a percentage of maximal response to ATP in the absence of inhibitor, which was defined as 100% response. The curves shown were calculated by non linear regression analyses. Hill values (n_H) were −0.89±0.12 (4) and −0.72±0.06 (4) for KN-62 and KN-04, respectively. Mean values from 4 experiments on 3 patients are shown; vertical lines indicate s.e.mean.

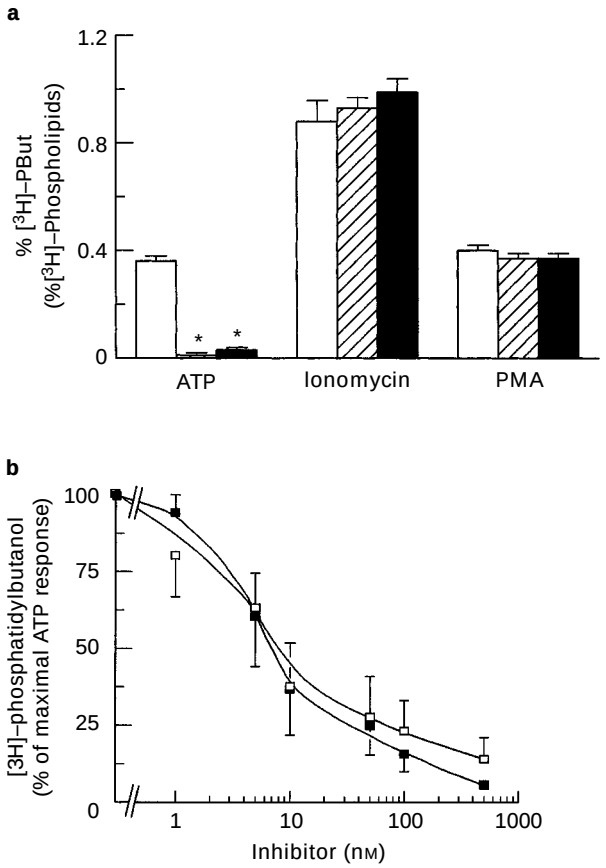

Figure 3 Effect of KN-62 and KN-04 on ATP-, ionomycin- or phorbol ester-stimulated phospholipase D (PLD) activity. [^{3}H]-oleic acid-labelled lymphocytes suspended in Ca^{2+}-containing (1 mM) HEPES-buffered KCl medium at 10^7 ml^{-1} were preincubated in the absence (open columns) or presence of KN-62 (hatched columns) or KN-04 (solid columns) for 5 min at 37°C, butanol (30 mM) was added for 5 min, then cells were stimulated for 15 min with (a) 1 mM ATP (ATP^{4-} 260 μM), 5 μM ionomycin or 100 nM phorbol ester (PMA). KN-62 and KN-04 were 500 nM. [^{3}H]-PBut was expressed as a percentage of total [^{3}H]-phospholipids (mean$\pm$s.e.mean, $n=3$) after subtraction of basal activity (0.09$\pm$0.01%). Significant difference in PLD activity: $*P<0.0001$ (ANOVA). Results shown are from one of two experiments (b) [^{3}H]-oleic acid-labelled lymphocytes were preincubated with or without KN-62 (■) or KN-04 (□) as detailed above and stimulated with 1 mM ATP. Results are expressed as a percentage of maximal response to ATP in the absence of inhibitor, which was defined as 100% response. The curves shown were calculated by non linear regression analyses. Hill (n_H) values were -1.03 ± 0.33 (3) and -0.75 ± 0.09 (3) for KN-62 and KN-04, respectively. Mean values of 3 patients are shown; vertical lines indicate s.e.mean.

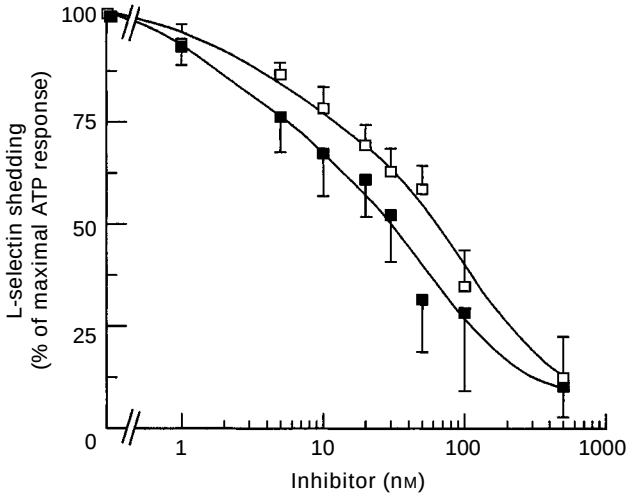

Figure 4 Inhibition of ATP-stimulated L-selectin shedding by KN-62 and KN-04. Lymphocytes, suspended at 10^6 ml^{-1} in Ca^{2+}-free HEPES-buffered KCl medium, were preincubated with KN-62 (■) or KN-04 (□) for 5 min at 37°C and stimulated with 500 μM ATP (ATP^{4-} 260 μM) for 5 min, then stained with FITC-labelled monoclonal anti-L-selectin antibody. The mean channel of cell-associated fluorescence was measured by flow cytometry. Results are expressed as a percentage of maximal response to ATP in the absence of inhibitor, which was defined as 100% response. The curves shown were calculated by non linear regression analyses. Hill (n_H) values were -0.93 ± 0.02 (3) and -0.84 ± 0.10 (3) for KN-62 and KN-04, respectively. Mean values of 3 patients are shown; vertical lines indicate s.e.mean.

stimulated L-selectin shedding with IC_{50} values of 31.5$\pm$4.5 and 78.7$\pm$10.8 nM ($n=3$), respectively (Figure 4).

Inhibition by KN-62 and KN-04 is time-dependent and partially reversible

KN-62 (15–100 nM) had little inhibitory effect on ATP-stimulated Ba^{2+} influx if both the inhibitor and the agonist (ATP, 640 μM) were added simultaneously to the lymphocyte suspension at zero time of the assay (data not shown). The slow onset of inhibitory action of the isoquinolinesulphonamides was best shown by measuring ATP-stimulated ethidium$^+$ influx. In the absence of inhibitor, the ATP-stimulated uptake of ethidium$^+$ was linear with time over 5 min but the addition of KN-62 or KN-04 (500 nM) three min after ATP (500 μM) produced a gradual slowing of the rate of ethidium$^+$ uptake to approximate the basal rate after 1 min.

The reversibility of KN-62 inhibition was examined by incubating cells with or without KN-62 (20 nM) for 10 min at 37°C, washing, and then measuring ATP-stimulated Ba^{2+} influx after the KN-62 washing procedure. The inhibitory effect of KN-62 was partially reversible, since the Ba^{2+} influx was restored to 50% of that observed for untreated cells when KN-62-treated cells were washed twice in medium containing 2% BSA. When 0.1% BSA-containing medium was used for washing the cells, 25% of P2Z-receptor activity was restored.

KN-62 and KN-04 do not inhibit the P2Y-mediated rise in cytosolic [Ca^{2+}]

The inhibitory effect of KN-62 and KN-04 on the G-protein-coupled, metabotropic P2Y receptors was studied in fresh human neutrophils. Both UTP (10 μM) (Figure 5) and ATP 10 μM) (results not shown) stimulated a rise in cytosolic [Ca^{2+}] from 210 nM to $\geqslant$900 nM in fura-2 loaded neutrophils incubated in either Ca^{2+}-containing (1 mM) or Ca^{2+}-free (plus 0.2 mM EGTA) saline medium. Peak values for the Ca^{2+} transient were >1 μM in Ca^{2+}-containing medium but were generally lower (ca. 0.9 μM) in Ca^{2+}-free medium (Figure 5). Neither KN-62 nor KN-04 (both 500 nM), had any effect on UTP- or ATP-stimulated rise in the cytosolic [Ca^{2+}].

Discussion

KN-62 and KN-04 are potent inhibitors of the P2Z-receptor

The main finding of this study is that KN-62 and KN-04 are potent inhibitors of the P2Z-receptor, since all P2Z-mediated responses in human lymphocytes were antagonized. Thus both KN-62 and KN-04 inhibited ATP-stimulated permeant fluxes (Figures 1 and 2) through the P2Z ion channel in a concentration-dependent manner over the same range (1–500 nM) that inhibited ATP-stimulated phospholipase D activity (Figure 3) and ATP-induced L-selectin shedding (Figure 4). While ATP-stimulated phospholipase D activity is directly dependent

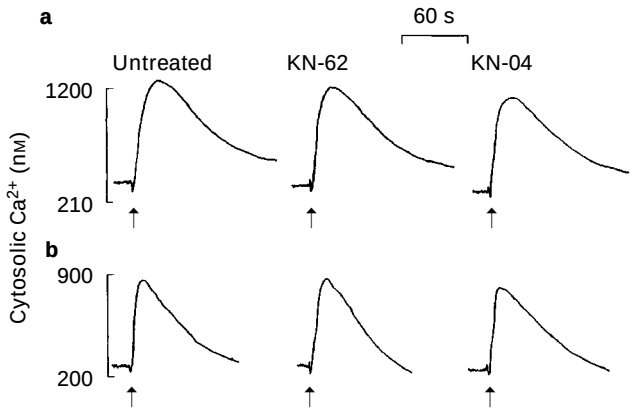

Figure 5 Effect of KN-62 and KN-04 on UTP-stimulated rise in cytosolic [Ca^{2+}] in human neutrophils, Fura-2 loaded neutrophils were suspended at 10^6 ml^{-1} in HEPES-buffered Ca^{2+}-free saline medium, preincubated with or without KN-62 or KN-04 (500 nM) for 5 min at 37°C in a stirred fluorimeter cuvette, and either (a) 1 mM Ca^{2+} or (b) 0.2 mM EGTA was added 1 min before stimulation with UTP (10 μM) as indicated. Results shown are from a single donor. Similar results were obtained from another donor.

on divalent cation influx through the P2Z-receptor operated ion channel (Gargett *et al*., 1996), ATP-induced L-selectin shedding is independent of these fluxes (Jamieson *et al*., 1996). These data show that both KN-62 and KN-04 directly inhibit the P2Z-receptor rather than simply blocking the associated ion channel. Furthermore, the specificity of KN-62 and KN-04 for P2Z-mediated responses was supported by the lack of effect of these compounds on ionomycin-stimulated rise in cytosolic [Ca^{2+}] (Gargett, Gu & Wiley, unpublished observations) and ionomycin-stimulated phospholipase D activity (Figure 3a). It is well documented that PMA, a membrane permeant phorbol ester activates protein kinase C, and stimulates both L-selectin shedding and phospholipase D activity (Kishimoto *et al*., 1989; Exton, 1994). However, neither of these responses, which do not involve P2Z-receptor activation, were inhibited by the isoquinolinesulphonamides (Figure 3a, Gargett, Gu & Wiley, unpublished observations).

P2Z-mediated responses are unlikely to involve CaMKII

This study shows that the inhibitory effect of KN-62 on the diverse effects mediated by ATP activation of P2Z-receptors present on human lymphocytes is not due to CaMKII inhibition. KN-62 has been extensively used as a protein kinase inhibitor, which is specific for CaMKII (IC_{50} of 0.9 μM), since even at 100 μM, this inhibitor has no effect on other protein kinases (Tokumitsu *et al*., 1990). In this study, we show that KN-62 inhibits both ATP-stimulated Ba^{2+} and ethidium$^+$ fluxes in a concentration-dependent manner with a potency (IC_{50} 13 nM), which is 100 fold greater than its inhibitory action on CaMKII (Figure 1 and 2). KN-04, a structural analogue of KN-62, with no inhibitory effect on CaMKII at 100 μM, has been used as a negative control in studies exploring the role of CaMKII. For example, KN-62 but not KN-04 inhibits the release of γ-aminobutyric acid (GABA) into the cerebrospinal fluid of the rat (Ishikawa *et al*., 1990), decreases the density of voltage-gated Na^+ channels in chick myotubes (Satoh *et al*., 1994), and inhibits both glucose-dependent release of insulin and cyclic-ADP-ribose-mediated Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores of rat pancreatic islets (Wenham *et al*., 1992; Takasawa *et al*., 1995). In all these studies it was concluded that CaMKII was involved in mediating these responses. In this study, KN-04 inhibited P2Z-mediated responses with IC_{50}s of the same order of magnitude as KN-62, which further suggests that CaMKII is unlikely to regulate the P2Z-receptor.

Specificity of isoquinolinesulphonamide antagonism

In contrast to the potent inhibition by KN-62 and KN-04 of the P2Z-receptor, these inhibitors had no effect on P2Y-receptors (Figure 5). While stimulation of P2Z- and P2Y-receptors results in a rise in cytosolic [Ca^{2+}], the mechanism by which this occurs is quite different. The P2Z-receptor is a ligand gated ion channel and conducts Ca^{2+} from the extracellular medium into the interior of the cell, without release of Ca^{2+} from intracellular stores (Dubyak & El-Moatassim, 1993; Gargett *et al*., 1996). In contrast, P2Y-receptors are G-protein-coupled and activate the phosphoinositide-specific phospholipase C signalling cascade releasing Ca^{2+} from internal stores (Dubyak & El-Moatassim, 1993, Figure 5). P2Y-receptors, present on human neutrophils, respond to ATP and/or UTP and at much lower concentrations (1–10 μM) than the concentration of ATP required to stimulate the P2Z-receptor. KN-62 and KN-04, at concentrations which completely inhibited all P2Z-mediated responses, had no effect on the $P2Y_2$-mediated rise in cytosolic [Ca^{2+}], whether the cells were suspended in Ca^{2+}-free or Ca^{2+}-containing medium (Figure 5). In other cell types which express $P2Y_2$ receptors, such as thyroid FRTL-5 cells (Dubyak & El-Moatassim, 1993) or biliary epithelial cells, KN-62 had no inhibitory effect on ATP-stimulated rise in cytosolic [Ca^{2+}] (McGill *et al*., 1995; Törnquist & Ekokoski, 1996). Thus the isoquinolinesulphonamide antagonists distinguish between P2Z- and P2Y-mediated responses.

Recent studies have shown that KN-62 and KN-04 have other effects and are equipotent inhibitors of voltage-gated Na^+ and Ca^{2+} channels, as well as receptor-operated Na^+ channels in bovine adrenal chromaffin cells (Marley & Thomson, 1996; Maurer *et al*., 1996; Tsutsui *et al*., 1996). However the action of the isoquinolinesulphonamides on these cation channels requires concentrations 2–3 orders of magnitude greater than those which inhibit the P2Z-mediated responses in this study. Furthermore, the maximal effect achieved with 10 μM KN-62 or KN-04 was 70–80% inhibition of voltage-gated $^{45}Ca^{2+}$ or $^{22}Na^+$ fluxes (Maurer *et al*., 1996; Tsutsui *et al*., 1996), whereas in the present study, 500 nM concentrations of these inhibitors blocked Ba^{2+} or ethidium$^+$ influx through the P2Z ion channel by 91–98% (Figure 1 and 2). However, KN-62 inhibition of the P2Z-receptor and the subsequent lack of cation fluxes is probably responsible for inhibition of ATP-stimulated phospholipase D activity, which is Ca^{2+} influx-dependent (Gargett *et al*, 1996).

KN-62 is readily membrane permeant as shown by its ability to inhibit intracellular CaMKII (Hidaka & Kobayashi, 1992). It is also likely that KN-04 is membrane permeant, since its structure is almost identical to KN-62 and slightly more hydrophobic. It is possible that these isoquinolinesulphonamides act intracellularly, conferring specificity by binding to the long intracellular carboxyl tail found only on the P2Y/$P2X_7$-receptor (Surprenant *et al*., 1996). This might explain why a 5–15 min preincubation is required to achieve maximal inhibition with KN-62 and KN-04, and also why reversibility of their inhibitory action is only partial after washing with media containing bovine serum albumin. Longer preincubations (30–60 min) are necessary for KN-62 antagonism of CaMKII (Wenham *et al*., 1992; Takasawa *et al*., 1995) compared to inhibition of the P2Z receptor, perhaps due to the higher intracellular concentration of inhibitor required for CaMKII inhibition. However, it is also possible that both KN-62 and KN-04 bind to either the putative large external region of the P2Z/$P2X_7$-receptor or close to one of the transmembrane regions and prevent ATP-induced conformational changes associated with ion channel opening.

This study shows that KN-62 and KN-04 are the two most potent inhibitors of the P2Z-receptor described to date. KN-62 has an IC_{50} of 13 nM for inhibition of cation fluxes and almost completely inhibits (>95%) all responses mediated by extracellular ATP in human lymphocytes. Thus, KN-62 and KN-04 are 2–3 orders of magnitude more potent as inhibitors of the P2Z-receptor than are HMA, oxidized ATP, or suramin, all of

which require 30–300 μM concentrations for maximal effect (Wiley *et al*., 1993; 1994; 1996). Although isoquinolinesulphonamide inhibition of P2Z-mediated responses is essentially complete after a 5 min preincubation, we have noted that at low KN-62 concentrations a 15 min preincubation sometimes gave an additional 5–10% inhibition. In contrast oxidised ATP requires a one hour preincubation. Furthermore, the inhibitory effect of KN-62 and KN-04 on P2Z-mediated responses is almost complete (91–98%), unlike that of HMA which only blocks cation fluxes 70–80% at maximal effective concentrations.

Currently there are no antagonists that are specific for any single P2-receptor subtype or even that distinguish between ionotropic and metabotropic P2-receptors. Even, PPADS, which initially emerged as a P2X-selective antagonist (Lambrecht *et al*., 1992), has since been shown to inhibit $P2Y_1$-but not $P2Y_2$-receptors co-expressed on endothelial cells (Brown *et al*., 1995). Further studies will be necessary to determine if the isoquinolinesulphonamides are also inhibitors of P2X-receptors in addition to their specificity for the P2Z-receptor. Thus KN-62 should prove a useful tool in analysing purinergic responses in the many tissues which co-express several P2-receptor subtypes.

We thank Ben Gu for assistance for L-selectin measurements and Dr Phil Marley and Dr Jean Cornish for helpful discussions. This work was supported by a grant from the National Health & Medical Research Council of Australia (to J.S.W.) and an Australian Postgraduate Research Award (to C.E.G.)

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(Received October 24, 1996
Revised January 9, 1997
Accepted January 17, 1997)